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Use of Multiplex PCR To Identify *Staphylococcus aureus* Adhesins Involved in Human Hematogenous Infections

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We have developed a multiplex PCR procedure to determine the distribution of nine adhesin genes in *Staphylococcus aureus* isolates. Only genes encoding bone sialoprotein binding protein and fibronectin binding protein B were significantly associated with hematogenous osteomyelitis/arthritis and native-valve endocarditis, respectively, suggesting their involvement in hematogenous tissue infections.

Staphylococcus aureus is both a commensal and an extremely versatile pathogen. It causes superficial lesions, deep-seated and systemic infections, and toxemic syndromes (10). With the exception of toxemic syndromes, which are directly due to toxin production, *S. aureus* pathogenesis results from the combined action of a variety of factors (1, 17). *S. aureus* infection begins with bacterial adhesion to host tissues. *S. aureus* adhesins are grouped into a single family named microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (5). MSCRAMM involvement in *S. aureus* pathogenicity in human beings is poorly documented. Results obtained in experimental models suggest that *S. aureus* strains that specifically express receptors for fibrinogen and fibronectin are associated with infective endocarditis, while strains that produce receptors for bone sialoprotein, collagen, and fibronectin are associated with osteomyelitis and arthritis (2, 3, 9, 18, 20, 21, 23). However, these associations have not been confirmed in all relevant studies.

Staphylococcal MSCRAMMs can be detected with binding assays using purified matrix molecules (11), but several adhesins are redundant. For example, there are two fibronectin binding proteins (FnbpA and -B) and three receptors for fibrinogen (clumping factors A and B [ClfA and -B] and fibrinogen binding protein [Fib]). In addition, some MSCRAMMs bind to more than one matrix molecule (for example, FnbpA binds to both fibronectin and fibrinogen) (22). Finally, binding assays are expensive, owing to the high cost of purified matrix molecules. PCR has been used to detect staphylococcal adhesin genes (12, 13, 16), but multiplex PCR technology has not yet been used to study adhesin distribution in *S. aureus* isolates of various origins. We therefore developed a multiplex PCR procedure to examine the distribution of nine MSCRAMM genes in *S. aureus* isolates from patients with nasal colonization and with invasive infections associated with hematogenous seeding.

The nucleotide sequences of *ebpS* (encoding elastin binding protein), *eno* (encoding laminin binding protein), *cna* (encoding collagen binding protein), *fnbA* and *fnbB* (encoding fibronectin binding proteins A and B), *fib* (encoding fibrinogen binding protein), *clfA* and *clfB* (encoding clumping factors A and B), and *bbp* (encoding bone sialoprotein binding protein) obtained from GenBank (accession numbers U48826, AF065394, M81736, X95848, X62992, X72014, Z18852, AJ224764, and Y18653, respectively) were compared and evaluated by using Blast and ClustalX softwares to identify regions unique to each gene, with similar annealing temperatures. Genomic DNA was extracted from staphylococcal cultures (2) and used as an amplification template with the primers (Eurogentec, Seraing, Belgium) described in Table 1 in both simplex and multiplex PCRs. Two primer sets were prepared for multiplex PCR: PCR1 to amplify *bbp*, *cna*, *ebpS*, and *eno* and PCR2 to amplify *fnbA*, *fnbB*, *fib*, *clfA*, and *clfB*. The thermal cycling conditions included an initial denaturation step (5 min at 94°C) followed by 25 cycles of amplification (denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C). The reaction was terminated with a 10-min incubation step at 72°C. PCR products were analyzed by electrophoresis through 1% agarose gels (Sigma, Saint Quentin Fallavier, France).

A subset of 157 *S. aureus* isolates, identified as *S. aureus* by their abilities to coagulate citrated rabbit plasma and to produce a clumping factor, were selected for PCRs. These strains were collected from nose swabs of asymptomatic carriers or from clinical specimens of patients with native-valve endocarditis or hematogenous osteomyelitis and/or arthritis (7, 8); types of infection were defined in accordance with published criteria (4, 6).

We first examined the specificity of the newly designed primers by means of simplex PCRs. As no control strains were available for the nine genes, we randomly selected 17 *S. aureus* strains and tested each with the nine simplex PCRs. All amplicons were consistent with the predicted sizes (Table 1). Two randomly selected amplicons from each of the nine simplex PCRs were subjected to DNA sequencing to confirm their specificity and were 98 to 100% identical to the corresponding GenBank sequences. The 17 strains of *S. aureus* tested by simplex PCR were then tested by multiplex PCR and yielded the expected band patterns; no discrepancies between the two methods were observed.

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TABLE 1. Nucleotide sequences and anticipated amplicon sizes for the *S. aureus* gene-specific oligonucleotide primers used in this study[a]

Gene	Primer	Nucleotide sequence	Amplicon size (bp)	Multiplex PCR
bbp	BBP-1	AACTACATCTAGTACTCAACAACAG	575	1
	BBP-2	ATGTGCTTGAATAACACCATCATCT		1
cna	CNA-1	GTCAAGCAGTTATTAACACCAGAC	423	1
	CNA-2	AATCAGTAATTGCACTTTGTCCACTG		1
eno	ENO-1	ACGTGCAGCAGCTGACT	302	1
	ENO-2	CAACAGCATYCTTCAGTACCTTC		1
ebpS	EBP-1	CATCCAGAACCAATCGAAGAC	186	1
	EBP-2	CTTAACAGTTACATCATCATGTTTATCTTTG		1
fnbA	FNBA-1	GTGAAGTTTTAGAAGGTGGAAAGATTAG	643	2
	FNBA-2	GCTCTTGTAAGACCATTTTTCTTCAC		2
fnbB	FNBB-1	GTAACAGCTAATGGTCGAATTGATACT	524	2
	FNBB2	CAAGTTCGATAGGAGTACTATGTTC		2
fib	FIB-1	CTACAACTACAATTGCCGTCAACAG	404	2
	FIB-2	GCTCTTGTAAGACCATTTTCTTCAC		2
clfA	CLFA-1	ATTGGCGTGGCTTCAGTGCT	292	2
	CLFA-2	CGTTTCTTCCGTAGTTGCATTTG		2
clfB	CLFB-1	ACATCAGTAATAGTAGGGGGCAAC	205	2
	CLFB-2	TTCGCACTGTTTGTGTTTGCAC		2

[a] The nucleotide sequences of *ebpS* (encoding elastin binding protein), *eno* (laminin binding protein), *cna* (collagen binding protein), *fnbA* and *fnbB* (fibronectin binding proteins A and B), *fib* (fibrinogen binding protein), *clfA* and *clfB* (clumping factors A and B), and *bbp* (bone sialoprotein binding protein) were obtained from GenBank under their specific accession numbers (U48826, AF065394, M81736, X95848, X62992, X72014, Z18852, AJ224764, and Y18653, respectively).

All 157 clinical isolates tested by multiplex PCR were positive for *eno* and *clfB*. The other MSCRAMM genes were heterogeneously detected: 99% of the strains were positive for *clfA*, 80% were positive for *fib*, 58% were positive for *ebpS*, 43% were positive for *fnbB*, 36% were positive for *cna*, 28% were positive for *fnbA*, and 22% were positive for *bbp* (Table 2). One isolate was positive for eight of the nine MSCRAMM genes, 20 isolates were positive for seven genes, 67 were positive for six genes, 63 were positive for five genes, 5 were positive for four genes, and 1 was positive for three genes (median, six genes per isolate). Eighty-six of the 157 isolates harbored none of the fibronectin binding protein genes, while all of the isolates harbored at least two of the three fibrinogen receptor genes (*fib*, *clfaA*, and *clfB*). The minimal MSCRAMM gene equipment in this panel of 157 isolates comprised two genes encoding fibrinogen receptor (including clumping factor B in all strains) and the gene encoding laminin binding protein. This confirms previous reports that most *S. aureus* isolates harbor functionally redundant MSCRAMMs (16).

The MSCRAMM gene distribution did not differ between isolates associated with nasal colonization and those associated with invasive infections (endocarditis and osteomyelitis/arthritis; $P > 0.05$ for each gene comparison [chi square test]; Table 2). In contrast, two MSCRAMM genes were significantly associated with specific invasive infections: *bbp* was associated more with osteomyelitis/arthritis than with endocarditis (38 versus 11%; odds ratio = 5.2; $P = 0.006$), and *fnbB* was associated more with endocarditis than with osteomyelitis/arthritis (55 versus 32%; odds ratio = 0.4; $P = 0.046$) (Table 2). Previous studies have shown that strains expressing receptors for bone sialoprotein are associated with osteomyelitis and arthritis (19) and that strains bearing receptors for fibronectin (but not specifically FnbB) are associated with infective endocarditis (18). However, in our study, not all of the isolates associated with osteoarthritis and endocarditis harbored the *bbp* or *fnbB* gene, respectively (Table 2). Thus, in addition to possible host factors, this tropism of *S. aureus* may involve additional MSCRAMMs, such as receptors for fibrinogen (in endocarditis) and for fibronectin and/or collagen (in osteomyelitis and arthritis) or other virulence factors involved in bacterial persistence at the site of infection after tissue binding (3, 15, 21, 23). Indeed, inactivation of a single MSCRAMM gene is not

TABLE 2. MSCRAMM gene distribution among 157 *S. aureus* clinical isolates

Gene	No. (%) of isolates			Odds ratio (95% CI), *P* value[a]
	Nasal colonization ($n = 82$)	Native-valve endocarditis ($n = 88$)	Hematogenous osteomyelitis and/or arthritis ($n = 37$)	
bbp	17 (21)	4 (11)	14 (38)	5.2 (1.5–17.7), 0.006
cna	30 (37)	12 (32)	15 (41)	1.5 (0.6–3.8), 0.43
eno	82 (100)	38 (100)	37 (100)	NS
ebpS	45 (55)	23 (61)	23 (62)	1.1 (0.4–2.7), 0.88
fnbA	26 (32)	11 (29)	7 (19)	0.6 (0.2–1.7), 0.31
fnbB	34 (42)	21 (55)	12 (32)	0.4 (0.152–0.99), 0.046
fib	67 (82)	29 (76)	29 (78)	1.1 (0.4–3.3), 0.83
clfA	82 (100)	38 (100)	35 (95)	2.1 (1.6–2.6), 0.146
clfB	82 (100)	38 (100)	37 (100)	NS

[a] Odds ratios and the chi square test were used to simultaneously analyze the distribution of nine MSCRAMM genes among *S. aureus* isolates from patients with osteomyelitis and/or arthritis versus native-valve endocarditis. CI, confidence interval. NS, no significant difference.

sufficient to abolish strain virulence in an experimental model of hematogenous *S. aureus* infections (14, 15, 21). Combined investigation of MSCRAMM and toxin gene distribution in *S. aureus* isolates would permit better understanding of the complex process of tissue seeding during bacteremia.

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